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Pseudomonas aeruginosa lipopolysaccharide inhibits C*andida albicans* hyphae formation and alters gene expression during biofilm development

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Keywords: biofilm; *Candida*; glycolysis; hypha; lipopolysaccharide; *Pseudomonas*
Accepted 5 September 2012
DOI: 10.1111/omi.12006

SUMMARY

Elucidation of bacterial and fungal interactions in multispecies biofilms will have major impacts on understanding the pathophysiology of infections. The objectives of this study were to (i) evaluate the effect of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) on *Candida albicans* hyphal development and transcriptional regulation, (ii) investigate protein expression during biofilm formation, and (iii) propose likely molecular mechanisms for these interactions. The effect of LPS on *C. albicans* biofilms was assessed by XTT-reduction and growth curve assays, light microscopy, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). Changes in candidal hypha-specific genes (HSGs) and transcription factor *EFG1* expression were assessed by real-time polymerase chain reaction and two-dimensional gel electrophoresis, respectively. Proteome changes were examined by mass spectrometry. Both metabolic activities and growth rates of LPS-treated *C. albicans* biofilms were significantly lower ($P < 0.05$). There were higher proportions of budding yeasts in test biofilms compared with the controls. SEM and CLSM further confirmed these data. Significantly upregulated HSGs (at 48 h) and *EFG1* (up to 48 h) were noted in the test biofilms ($P < 0.05$) but cAMP levels remained unaffected. Proteomic analysis showed suppression of candidal septicolysin-like protein, potential reductase-flavodoxin fragment, serine hydroxymethyltransferase, hypothetical proteins Cao19.10301(*ATP7*), CaO19.4716(GDH1), CaO19.11135(*PGK1*), CaO19.9877(*HNT1*) by *P. aeruginosa* LPS. Our data imply that bacterial LPS inhibit *C. albicans* biofilm formation and hyphal development. The *P. aeruginosa* LPS likely target glycolysis-associated mechanisms during candidal filamentation.

INTRODUCTION

Lipopolysaccharides (LPS) are a major virulence factor and an integral component of the outer cell membrane of gram-negative bacteria. They provide barrier protection for the organisms from environmental stresses and antimicrobial agents. During host–microbial interactions, LPS actively mediate recognition, adhesion, colonization and survival of the organism

under extreme environments (Aurell & Wistrom, 1998; De Castro *et al.*, 2010). The LPS also play an important role in the pathogenesis of gram-negative infections by eliciting proinflammatory and immunomodulatory responses, which lead to septic shock in human and animal hosts (Rietschel *et al.*, 1982; Morrison & Ryan, 1987; Aurell & Wistrom, 1998; De Castro *et al.*, 2010).

In general, species belonging to the genus *Candida* are commensal dimorphic fungi that reside in the human gastrointestinal tract and genitourinary tract (Samaranayake, 2002; Miceli *et al.*, 2011). Oral *Candida* can be isolated from over one-third of healthy adults, while its prevalence in hospitalized patients may be as high as 55% (MacFarlane, 1990). An exceptionally high prevalence has been documented from oral samples taken from the dorsal tongue surface in the Japanese population (67%), and its presence in the oral cavity has been significantly associated with dental prostheses, missing teeth, retained roots and periodontal pockets (Wang *et al.*, 2006). When host immunity is impaired locally or systemically, *Candida* becomes an opportunistic pathogen and invades the host. This may manifest as commonly seen superficial *Candida* infections and less common systemic, life-threatening candidemia (Leroy *et al.*, 2009). ARTEMIS Global Antifungal Surveillance Program has reported that *C. andida albicans* is the most common causative agent of systemic candidal infections (63–70%), followed by *Candida glabrata* (44%), *Candida tropicalis* (6%) and *Candida parapsilosis* (5%) (Pfaller *et al.*, 2007).

Biofilms formed by both single and multiple species are responsible for almost 70% of human microbial infections (Lewis, 2001). Versatility in adaptation to different habitats, and the formation of biofilms are well-known factors that augment microbial virulence (Ramage *et al.*, 2005). As most biofilms are polymicrobial communities, interspecies interactions may either accentuate or suppress human infections (Douglas, 2003; Shirtliff *et al.*, 2009b; Morales & Hogan, 2010). Hence, *Escherichia coli* increases the probability of fungal urinary tract infections by promoting the adhesion of *C. albicans* to bladder mucosa (Levison & Pitsakis, 1987), whereas *C. albicans* increases the risk of ventilator-associated pneumonia caused by *Pseudomonas aeruginosa* (Pierce, 2005; Azoulay *et al.*, 2006). The interactions of various oral bacteria including *Streptococcus*, *Actinomyces* and *Fusobacterium* with *C. albicans* may contribute to oral candidiasis (Bamford *et al.*, 2009; Shirtliff *et al.*, 2009b). In contrast, lactobacilli in the female reproductive system inhibit *C. albicans* attachment and invasion (Hogenauer *et al.*, 1998).

Interactions within such mixed species biofilms may be mediated by protein–protein, lectin–carbohydrate, hydrophobic–electrostatic, cell surface molecules (e.g. glycoproteins and agglutinin-like sequences), quorum sensing and endotoxins (e.g. bacterial LPS) (Federle & Bassler, 2003; Hogan *et al.*, 2004; Klotz *et al.*, 2007; Shirtliff *et al.*, 2009b; Bandara *et al.*, 2010a; Morales & Hogan, 2010).

Though various molecules involved in bacterial–candidal interactions have been extensively studied, the effects of LPS on *Candida* have been sparsely investigated. Palma *et al.* (1992) demonstrated that LPS from *E. coli*, *Salmonella typhimurium* and *Serratia marcescens* strongly enhanced the inhibitory response of human polymorphonuclear leukocytes against the *in vitro* growth of *C. albicans*. In addition, LPS from *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *S. marcescens* and *Salmonella typhimurium* were shown to directly modulate *in vitro Candida* spp. biofilm formation in a species-specific and time-dependent manner (Bandara *et al.*, 2009, 2010a). Another study conducted by Henry-Stanley *et al.* (2003) demonstrated that LPS from *E. coli* augments the virulence of parenterally administered *C. albicans.* Although the effects of various secretory and structural components of bacteria affecting *Candida* biofilms have been well established, the specific roles of bacterial LPS in hyphal development and proteome regulation in *Candida* biofilms are yet to be determined.

Hence, the aims of this study were to evaluate the effect of LPS derived from *P. aeruginosa*, on *C. albicans* hyphal development, transcriptional regulation and protein expression during biofilm formation using growth, ultrastructural, genomic and proteomic assays. Furthermore, we aimed to propose a probable molecular mechanism(s) by which bacterial LPS modulate/inhibit *C. albicans* hyphae formation during biofilm development. *Pseudomonas aeruginosa* has been selected as the source of LPS because of its well-known association with *C. albicans* biofilm pathologies (Pierce, 2005).

METHODS

Microorganisms

Candida albicans ATCC90028, *C. albicans* SC5314 and a clinical strain of *C. albicans* from the archival *Candida* culture collection in the Oral Biosciences Laboratory, Faculty of Dentistry, The University of Hong Kong were used in this study. Microbial identity was confirmed with commercially available API 32 C for *Candida* strains (Biomérieux, Mercy l'Etoile, France). All isolates were stored in multiple aliquots at –20°C, after confirming their purity.

Lipopolysaccharides

Lipopolysaccharides purified from *K. pneumoniae* (Catalog No.L4268, Total impurities < 3%) and *P. aeruginosa* (Catalog No. L9143, Total impurities < 3%) were purchased in the form of lyophilized powder from Sigma Aldrich (St. Louis, MO) and stored 2–8°C until use.

Growth media

Sabouraud Dextrose Agar and Yeast Nitrogen Base (YNB) solution supplemented with 100 mM glucose were used for culturing *C. albicans*.

Microbial inocula

Before each experiment, *C. albicans* strains were subcultured on Sabouraud Dextrose Agar for 18 h at 37°C. A loopful of overnight *C. albicans* growth was inoculated into YNB medium and incubated for 18 h in an orbital shaker (75 r.p.m.) at 37°C. The resultant culture was harvested, washed twice in phosphate-buffered saline (PBS, pH 7.2) and resuspended. Cell suspensions were adjusted to 1×10^7 cells ml^{-1} by spectrophotometry and confirmed by hemocytometric counting.

Biofilm formation

Candida albicans biofilms were developed as described by Bandara *et al.* (2009) with some modifications. Commercially available pre-sterilized, polystyrene, flat-bottom 96-well microtiter plates (IWAKI, Tokyo, Japan) were used. A standard cell suspension (100 μl) of *C. albicans* (10^7 organisms ml^{-1}) was prepared and transferred into the wells of a microtiter plate, and incubated for 1.5 h (37°C, 75 r.p.m.) to promote microbial adherence to the surface of the wells. After the initial adhesion phase, the cell suspensions were aspirated and each well was washed twice with PBS to remove loosely adherent cells. A total of 200 μl YNB was transferred to each well and the plate was re-incubated for 48 h (37°C, 75 r.p.m.), and wells were washed twice with PBS to eliminate traces of the medium. Hence, the effect of bacterial LPS on *C. albicans* was studied over a 48-h period.

Quantitative analyses

Evaluation of the impact of LPS on C. albicans – XTT reduction assay

To support the hypothesis that bacterial LPS quantitatively affect *C. albicans* biofilms, an XTT reduction assay was performed on both LPS-treated and control biofilms at a defined time point. The metabolic activity of the biofilm cells was measured as a reflection of the viable cell counts of *C. albicans* biofilms.

Lipopolysaccharides of *K. pneumoniae* and *P. aeruginosa* (1 mg ml^{-1}, Sigma Aldrich) were serially diluted with YNB supplemented with 100 mM glucose (LPS concentrations 100 μg ml^{-1} to 1 ng ml^{-1}, test medium). The LPS was replaced with sterile PBS in the control samples. The *C. albicans* biofilms were developed in 96-well plates for 48 h to evaluate the effect of LPS as described below.

First, a *C. albicans* suspension (100 μl each) was dispersed into selected wells of a 96-well plate at time 0 and incubated in an orbital shaker (75 r.p.m.) at 37°C for 90 min for the adhesion phase. Subsequently, wells were washed twice with sterile PBS, and 200 μl of test and control media was added to pre-adherent *C. albicans*, and incubated for 48 h (37°C, 75 r.p.m.) for *C. albicans* biofilm formation. The supernatant was removed, and wells were washed twice with sterile PBS and a standard XTT reduction assay was performed as described by Jin *et al.* (2004) to measure the biofilm metabolic activity. In brief, commercially available XTT powder (Sigma) was dissolved in PBS at a final concentration of 1 mg ml^{-1} and filter-sterilized (0.22-μm pore size filter). Thereafter, 158 μl PBS, 40 μl XTT and 2 μl freshly prepared menadione solution (0.4 mM) were added into each well containing prewashed biofilms and incubated in the dark for 3 h

at 37°C. Color changes were measured with a microtitre plate reader (Spectra Max 340 tunable microplate reader; Molecular Devices Ltd, Sunnyvale, CA) at 492 nm. All assays were carried out in triplicate on three different occasions.

Qualitative analyses

Confocal laser scanning microscopy (CLSM) (Jin *et al.*, 2005) and scanning electron microscopy (SEM) were used to observe the ultrastructure and the variations in hyphal development of test and control *C. albicans* biofilms.

Confocal laser scanning microscopy

Commercially available sterile flat-bottom six-well plates (Iwaki Glass Co. LTD, Chiba, Japan) and plastic coupons (Thermanox plastic cover slips; Nulge Nunc International, Rochester, NY) (Ramage *et al.*, 2001) were used to prepare biofilms (both control and LPS-treated) as described above. Coupons were placed in wells of six-well plates; *C. albicans* suspensions were added and incubated in an orbital shaker (75 r.p.m.) at 37°C for 90 min for the adhesion phase. Test and control media were added at 90 min to pre-adherent *C. albicans* and incubated for 48 h for the biofilm formation phase. Prewashed coupons were stained with Live and Dead stain (Live/Dead BacLight Bacterial Viability kit; Invitrogen, Eugene, OR) (Jin *et al.*, 2005), and biofilms were analysed by fluorescence microscopy under CLSM.

Scanning electron microscopy

Biofilms (control and LPS-treated) were cultured on custom-made, tissue-culture-treated, polystyrene coupons as described above for CLSM. At designated time points, selected coupons were removed from wells, washed twice with PBS and placed in 1% osmium tetroxide for 1 h. Samples were washed in distilled water, dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, and 100% for 20 min), and air-dried in a desiccator before being sputter-coated with gold. Specimens were then mounted on aluminum stubs with copper tape, and coated with gold under low-pressure with an ion sputter coater (JEOL JFC1 100: JEOL, Tokyo, Japan). The surface topographies of the biofilm were visualized under SEM (Philip XL30CP) in high-vacuum mode at 10 kV.

Real-time PCR assay

Real-time polymerase chain reaction (PCR) was performed to assess the effect of *P. aeruginosa* and *K. pneumoniae* LPS on differential expression of *C. albicans* hypha-specific genes (HSGs) and the transcription factor *EFG1*. A 50-μM farnesol (Shirtliff *et al.*, 2009a; Weber *et al.*, 2010) solution was used as a positive control.

Candida albicans SC5314 biofilms were treated with LPS (test) and controls (media, farnesol) at 90 min and samples were collected at 1 h, 3 h, 6 h, 12 h, 24 h and 48 h. These time-points were selected to represent different stages of *C. albicans* biofilm formation. The times 1 h and 3 h were selected for irreversible adhesion and proliferation, 6 h for microcolony formation, 12 h and 24 h for initial colonization and extracellular matrix synthesis and 48 h for mature biofilms. RNA was extracted using the SV total RNA isolation system (Promega, Madison, WI, Catalog No. Z3100) and 2 μg template was reverse transcribed with Superscript II (Invitrogen). Resultant cDNA was amplified by PCR and specificity of the product was confirmed by sequencing.

Real-time PCR assays were performed to quantify the expression of HSGs at 48 h and of *EFG1* at all given time points. Real time PCR primers were designed for *C. albicans* HSGs and *EFG1* (Table 1) using PRIMER EXPRESS software (Version 3.0, Applied Biosystems, Carlsbad, CA). Primers were designed in such a way that amplicon size and T_m remained between 50 and 150 base pairs (bp) and 58 and 60°C, respectively. Real-time PCR contained Fast SYBR Green Master Mix 10 μl, reverse and forward primers (1 μl each) and sterile MiliQ water (7 ml) to make final volumes of 20 μl. The PCR were run on Step One Real-time PCR systems (Version 2, Applied Biosystems) (95°C incubation for 20 s, followed by 40 cycles of 95°C incubation for 1 s and 60°C for 20 s). Each primer pair produced a single amplicon with a uniform melting curve. A standard curve was constructed with the series of purified PCR products, and the absolute copy number of amplicons was quantified (amplification efficiency >90%, $R^2 > 0.970$). *EFB1* was used as the housekeeping (reference) gene (Schaller *et al.*, 1998) and all experiments were carried out in duplicate on three different occasions. Gene expression data (according to Applied Biosystems Real-time PCR guide, fluorescence intensity is

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

	Primer	Sequence
Hypha-specific gene		
ECE1	Forward primer	GTCGTCAGATTGCCAGAAATTG
	Reverse primer	CTTGGCATTTTCGATGGATTGT
HYR1	Forward primer	TTGTTTGCGTCATCAAGACTTTG
	Reverse primer	GTCTTCATCAGCAGTAACACAACCA
RBT4	Forward primer	GTGGCTCCTCTTCTGGTAGTAATGA
	Reverse primer	GCATCCAAGATTTGTTGAGCAA
RBT1	Forward primer	CCATCAACAAGATCAAACAAAATACA
	Reverse primer	CGAGTTGGGCAGTTGCAAA
ALS8	Forward primer	CCCTGAGTCCGCCATTTTT
	Reverse primer	ACTTTCTTATGCACGATTTATTTCCA
HWP1	Forward primer	GCCCAGAAAGTTCTGTTCCA
	Reverse primer	TTTGGTTTCAGTAGTAGTGGTTGG
ALS3	Forward primer	CTGGACCACCAGGAAACACT
	Reverse primer	ACCTGGAGGAGCAGTGAAAG
Transcription factor		
EFG1	Forward primer	TGTGAAAATCGGATCAATGCA
	Reverse primer	CATGGCCAATGCTCTTTCAA
Protein spot		
Septicolysin-like protein	Forward primer	GAATTGGCTAAATTGGTATCATCA
[*Candida albicans* SC5314]	Reverse primer	TCGGTCTCGACAAATTAATGC
Hypothetical protein CaO19.10301	Forward primer	GCTAAACAAGCTGCCAGAAAAGTT
[*Candida albicans* SC5314]	Reverse primer	CATCATGTCTTTTTTTGAATGCAGTT
Hypothetical protein CaO19.4716	Forward primer	GCCATCTGCTACCCAAAACG
[*Candida albicans* SC5314]	Reverse primer	TTACAACCAGCATCAACCAAAGC
Hypothetical protein CaO19.11135	Forward primer	TTGATCTTGCCAGTTGATTTTGTC
[*Candida albicans* SC5314]	Reverse primer	GCATCAGTAGCAGAAGAAGTTTTGG
Hypothetical protein CaO19.9877	Forward primer	AATTGCTGAAGCCCACGTTT
[*Candida albicans* SC5314]	Reverse primer	CGTCTGGAATGTTGTGCAACTT
Serine hydroxymethyltransferase, mitochondrial	Forward primer	TGTCCGATATGGCCCACAT
OS = *Candida albicans* GN = *SHM1* PE = 3 SV = 1	Reverse primer	TGGGAATGGTGAATCAGTAACAAC

The primer sequences used for quantification of differential expressions of hypha-specific genes, *EFG1* and the genes that encoded proteins unexpressed in *Pseudomonas aeruginosa* lipopolysaccharide-treated *C. albicans* SC5314 biofilms by quantitative real-time polymerase chain reaction.

proportional to the double-stranded PCR products produced) were normalized with EFB expression and statistically analysed.

Enzyme-linked immunosorbent assay

As cAMP acts upstream of *EFG1* and controls the cAMP/PKA pathway that regulates HSG expression, cAMP competitive enzyme-linked immunosorbent assay (ELISA; a competitive immunoassay for the quantitative determination of cAMP), was performed using 100 μg ml^{-1} *P. aeruginosa* and *K. pneumoniae* LPS, and 50 μM farnesol-treated *C. albicans* SC5314 biofilms at 12 h, 24 h and 48 h.

As the kit was originally designed for mammalian cells and bacteria (cAMP competitive ELISA, EMSCAMP kit, Thermo Scientific, Pierce Biotechnology, Rockford, IL), lysis of yeast samples was performed using Y-PER yeast protein extraction reagent (catalog No.78991, Thermo Scientific, Pierce Biotechnology, Rockford, IL). Briefly, both test and control *C. albicans* biofilms grown in tissue culture-treated polystyrene well plates were washed and resuspended in PBS (10^7 cells ml^{-1}) at three predefined time points. The samples were centrifuged (3000 ***g***, 5 min, 4°C) and pelleted. Cells were resuspended in appropriate amounts of Y-PER reagent and agitated for 20 min at room temperature. Cellular debris was pelleted again by centrifugation (14,000 ***g***, 10 min) and the supernatant was kept for further analysis.

Cyclic AMP competitive ELISA was performed according to the manufacturer's instructions. The

samples, standards and other required agents were added to designated wells followed by the addition of cAMP-AP conjugates and cAMP antibody. The plate was incubated at room temperature on a plate shaker for 2 h at 500 r.p.m. Wells were emptied, washed thrice with wash buffer, and tapped firmly to remove residual buffer. Thereafter, p-nitrophenyl phosphate substrate solution was added to every well and incubated at room temperature for 45 min without shaking. Stop solution was added and the plate was read immediately [optical density (OD) at 405 nm with a correction of 570 nm].

The data were analysed by a weighted four-parameter logistic curve. The average Net OD bound for each standard and sample was calculated (Average Net OD = Average Bound OD − Average non-specific binding OD). The Percent Bound of each pair of standard wells as a percentage of the maximum binding wells was also calculated (B_0) where% Bound = [(Net OD/Net B_0 OD) × 100]. The% Bound (B/B_0) vs. Concentration of cAMP for the standards was plotted and the concentration of cAMP in the samples was determined by interpolation.

Protein expression analysis

Protein extraction from yeasts and purification

Standard suspensions of *C. albicans* SC5314 were added to a six-well plate and incubated (75 r.p.m., 37°C, orbital shaker) for 90 min for the adhesion phase. Wells were washed twice with sterile PBS and 100 μg ml^{-1} *Pseudomonas* LPS in YNB medium (Test) and YNB medium alone (Control) were added. Plates were incubated for another 18 h to observe maximum differences in growth (Fig. 1), and washed twice with PBS before collection of samples for protein extraction.

Yeast suspensions were centrifuged and resuspended in ammonium carbonate (1.89 g ml^{-1}) with 1% β-mercaptoethanol (Sigma Aldrich). Suspensions were transferred to micro test tubes containing 1 g sterile glass beads and vigorously vortexed for 1 min and left 1 min in the ice. This process was repeated for seven or eight cycles. Suspensions were centrifuged for 15 min at maximum speed (20878.65 ***g***) at 4°C, and supernatants were collected immediately and stored at − 80°C. The protein-containing supernatants were subjected to Ready-Prep 2-D Clean up Kit (Bio-Rad, Hercules, CA) and stored at − 80°C. Purified proteins were quantified with the 2-2-D Quant kit (GE health care life sciences, Pittsburgh, PA).

First-dimension electrophoresis

Extracted protein was solubilized and diluted (10 mg ml^{-1} approximately) with lysis buffer (Urea 4.2 g, Thiourea 1.52 g, CHAPS 0.2 g, dithiothreitol 0.0154 g, IPG buffer 50 μl, MiliQ water up to 10 ml)

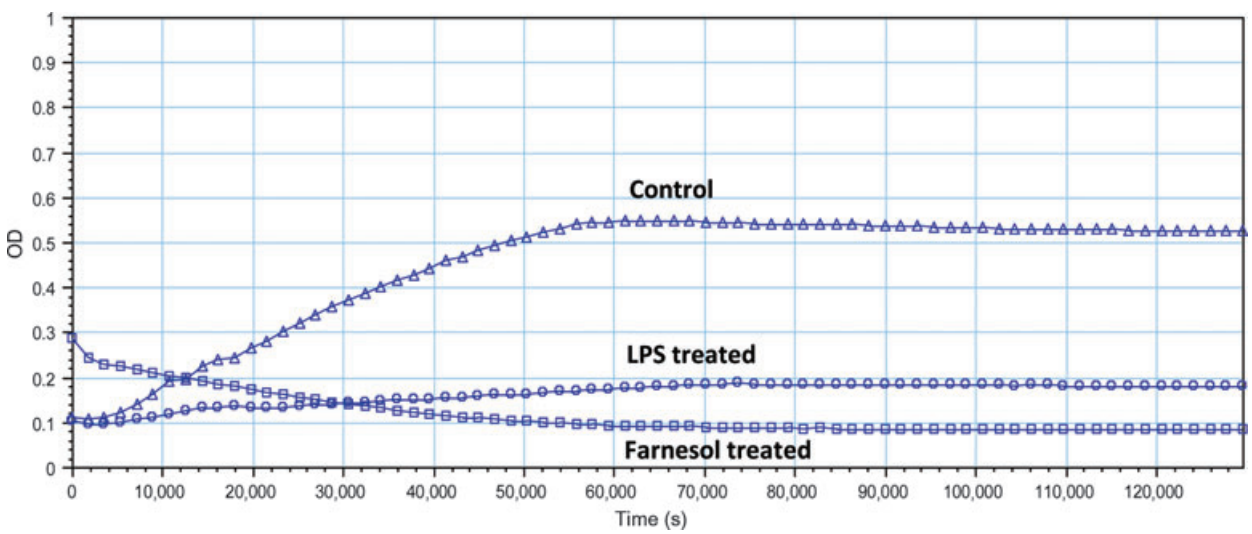

Figure 1 *Candida albicans* SC 5314 growth curve for 36 h. Growth curve assay for *Pseudomonas* lipopolysaccharide (LPS; 100 μg ml^{-1}, circles), farnesol (50 μM, squares) treated and control (triangles) *C. albicans* SC5314 for 36 h. Note the suppression of cell density (optical density) of LPS-treated sample compared with *C. albicans* control. Maximum difference was achieved around 18 h of incubation. *y*-axis represents optical density (OD) and each 10,000 s approximately equal to 2.8 h.

and pH 3–10 IPG strips were added and rehydrated at room temperature overnight. First-dimensional IEF was carried out in Protein IEF Cell (Bio-Rad, USA) over night (Step 1 at 250 V for 15 min; Step 2 at 10,000 V for 3 h; Step 3 elevation from 10,000 V to 70,000 V; Step 4 at 70,000 V; Step 5 at 500 V/Hold).

Second-dimension electrophoresis

After first-dimension electrophoresis, the IPG gel strips were equilibrated (equilibration buffer I and II, Bio-Rad Laboratories) and carefully placed on top of 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel with a protein marker on one side (precision plug protein, Bio-Rad Laboratories). The second-dimension electrophoresis was performed in PROTEAN II xi Cell (Bio-Rad Laboratories). The SDS–PAGE gel was fixed overnight, silver stained, scanned with calibrated densitometer (GS 800, Bio-Rad Laboratories) and analysed with two-dimension analysis software (PD QUEST; Bio-Rad Laboratories).

Image analysis

Briefly, at least three technical and two biological replicates (gels) were prepared, and scanned for both control and *P. aeruginosa* LPS-treated *C. albicans* samples. Spots were detected under automatic settings and rechecked manually to remove background noise and artifacts. Consistent and reproducible protein spots were further analysed. The spots that were absent in either test or control samples (in all replicates) were considered as differentially expressed proteins in *C. albicans* biofilms in response to *P. aeruginosa* LPS. Significant spots were excised and destained.

In-gel digestion of peptides was performed with trypsin (12.5 ng μl^{-1}, Promega) and peptides were extracted by Zip-Tip procedure (Millipore Corporation, Billerica, MA).

Tandem mass spectrometry

Protein samples were subjected to tandem mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy/mass spectroscopy (MALDI TOF MS/MS) was performed with a Bruker Autoflex III MALDI TOF/TOF Mass Spectrometer (Bruker Daltonics, Bremen, Germany) and Dionex UltiMate 3000 nano-LC system using a 50-Hz frequency laser beam. The following parameters were used for identification of candidate proteins in the NCBInr 20100408 (10820686 sequences; 3689795467 residues) (Taxonomy: Fungi, 674475 sequences) database using MASCOT software (http://www.matrixscience.com/); Type of search: MS/MS Ion Search, Enzyme: Trypsin/P, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Mass values: Monoisotopic, Protein Mass: Unrestricted, Peptide Mass Tolerance: ±50 ppm, Fragment Mass Tolerance: ±0.5 Da, Max Missed Cleavages: 1, Instrument type: MALDI-TOF-TOF. Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits at a significance level of 0.05. Identified proteins (named according to the NCBInr database) were functionally characterized, and encoding genes were determined using the Candidagenome database (http://www.candidagenome.org/, Stanford Genome Technology Centre), NCBI database (http://www.ncbi.nlm.nih.gov/) and, SWISSPROT and TrEMBL non-redundant protein database (http://www.expasy.ch/sport/).

Gene expression analysis with quantitative real-time PCR

Real-time PCR was performed to evaluate the relative expression of the genes encoding the downregulated proteins after Pseudomonas LPS treatment. *Candida albicans* SC5314 test and control samples were prepared as mentioned under protein extraction from yeast. RNA was extracted, reverse transcribed as mentioned under Real-time PCR assay.

Real-time PCR primers were designed for the genes coding for *C. albicans* downregulated protein spots (Table 1) and real-time PCR was performed as described above. All experiments were carried out in duplicates on three different occasions.

Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0). Mann–Whitney *U*-test was performed to compare the control and each test samples of the *C. albicans* biofilm. The relative gene expression data from real-time PCR and data from ELISA were analysed by Mann–Whitney *U*-test. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Bacterial LPS alters *C. albicans* biofilm metabolism in a strain-dependent and concentration-dependent manner

After 48 h of *P. aeruginosa* and *K. pneumoniae* LPS treatment (with 100 μg ml^{-1} and 10 μg ml^{-1} LPS), biofilm metabolism of the *C. albicans* ATCC strain was significantly enhanced (Fig. 2, see Tables S1 and S2). In contrast, however, adding 100 μg ml^{-1} of either bacterial LPS to the clinical and SC5314 strains produced a significant suppression of biofilm metabolism ($P < 0.05$). Enhanced biofilm metabolism was observed with the addition of lower concentrations of LPS for the *C. albicans* clinical strain [*Klebsiella* LPS (1 μg ml^{-1}) and *P. aeruginosa* LPS (10 μg ml^{-1} and 1 μg ml^{-1})] and *C. albicans* SC5314 strain [*Klebsiella* LPS (10 μg ml^{-1} and 1 μg ml^{-1}) and *P. aeruginosa* LPS (1 μg ml^{-1})]; ($P < 0.05$) (Fig. 2, see Tables S1 and S2). However, the effect of *P. aeruginosa* LPS on the metabolic activity of *C. albicans* biofilms was slight and strain specific. The *C. albicans* clinical strain and SC5314 strain behaved in a somewhat similar manner in the presence of LPS (Fig. 2, see Tables S1 and S2).

Maximum growth difference between LPS-treated *C. albicans* SC5314 and its control was observed at 18 h (Fig. 1). The reduction in optical density accounts for the suppression of both hyphae and yeast development.

Pseudomonas LPS affects *C. albicans* biofilm architecture

Confocal laser scanning microscopy

The CLSM with 'Live and Dead stain' indicated that *C. albicans* SC5314 48-h biofilms treated with *P. aeruginosa* LPS were scanty, and mainly consisted of patchy growth of blastospores (Fig. 3B). In contrast, the *C. albicans* control biofilms were well-structured with profuse hyphae (Fig. 3A). There were no dead cells and no biofilm matrix visible in both samples.

Scanning electron microscopy

After 48 h of incubation, *C. albicans* SC5314 biofilm controls demonstrated a densely colonized, highly organized profile with relatively long, intermingled hyphal elements. Extracellular matrix was scarcely visible under SEM (Fig. 3C). Supporting the CLSM findings, *C. albicans* treated with *Pseudomonas* LPS showed a scanty biofilm with a higher proportion of blastospores. Hyphae were fewer in number and shorter (Fig. 3D).

Pseudomonas LPS significantly upregulates expression of HSGs

Real-time PCR data for differential expression of *C. albicans* SC5314 hypha-specific genes at 48 h is shown in Fig. 4 and in Table S3. Expression was significantly upregulated after 48 h in both *P. aeruginosa* and *K. pneumoniae* LPS-treated samples for all seven HSGs tested ($P < 0.05$) (Fig. 4, Table S3). Farnesol significantly downregulated the expression of all HSGs except *HYR1* ($P < 0.05$) (Fig. 4, Table S3). Farnesol is known for the inhibition of *Candida* hyphae formation and was used as a control compound.

The expression of transcription factor *EFG1* in *C. albicans* SC5314 biofilms was significantly upregulated by *P. aeruginosa* LPS at all six time points tested, whereas *K. pneumoniae* LPS also demonstrated a similar effect except at the 3-h time point ($P < 0.05$) (Fig. 5, Table S4). In contrast, farnesol significantly downregulated *EFG1* during the whole course of *C. albicans* SC5314 biofilm formation ($P < 0.05$) (Fig. 5, Table S4).

Candida albicans cAMP levels are not affected by *Pseudomonas* LPS treatment

Data from cAMP competitive ELISA were plotted as percent bound against cAMP concentration, and did not show any significant differences (Table 2).

Pseudomonas LPS inhibits protein synthesis in *C. albicans* biofilms

Analysis of two-dimensional gels for the proteome of *C. albicans* SC5314 biofilms treated with *P. aeruginosa* LPS identified seven protein spots that were absent in LPS-treated biofilms compared with the control (Fig. 6). The MALDI-TOF MS and MS/MS data revealed that these spots represented proteins that acted as virulence factors, and partook in metabolic pathways, cell division and apoptosis.

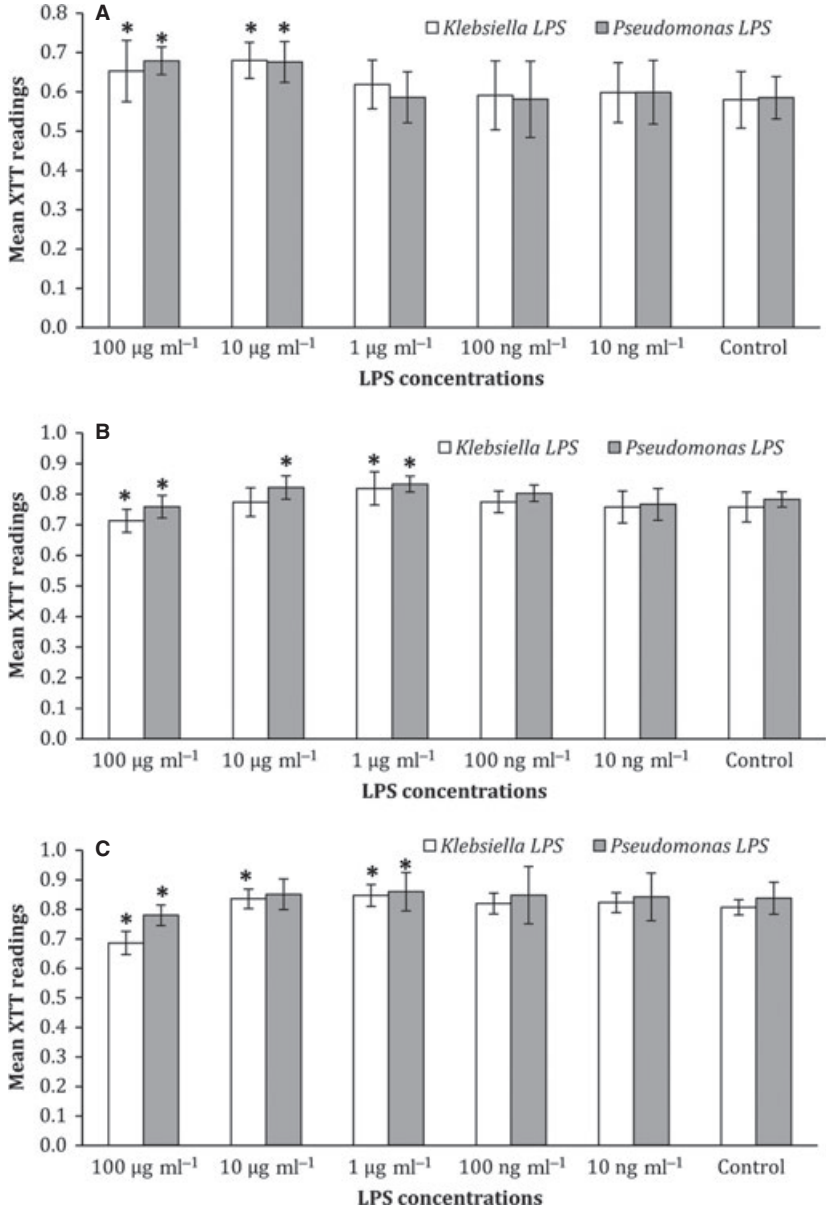

Figure 2 The mean XTT values (±SD) of *Candida albicans* control and test biofilms treated with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* lipopolysaccharide (LPS) in a concentration gradient for 48 h. The Mean XTT values of *Candida* spp. biofilms treated with *P. aeruginosa* and *K. pneumoniae* LPS in a concentration gradient (100 μg ml^{-1} to 10 ng ml^{-1}) for 48 h. $P < 0.05$ was considered statistically significant. (A) Effect of bacterial LPS on *C. albicans* ATCC90028 biofilms (90 min to 48 h); significantly enhanced mean XTT values were noted in *C. albicans* ATCC90028 biofilms treated with 100 and 10 μg ml^{-1} of both bacterial LPS ($P < 0.05$). (B) Effect of bacterial LPS on *C. albicans* clinical strain biofilms (90 min to 48 h); significantly inhibited mean XTT values were observed in *C. albicans* clinical strain biofilms treated with 100 μg ml^{-1} bacterial LPS while treatment of 10 μg ml^{-1} and 1 μg ml^{-1} of *P. aeruginosa* LPS, and 1 μg ml^{-1} of *K. pneumoniae* LPS resulted in significantly enhanced mean XTT values ($P < 0.05$). (C) Effect of bacterial LPS on *C. albicans* SC5314 biofilms (90 min to 48 h); a significant reduction of mean XTT values was noted in *C. albicans* SC5314 biofilms treated with 100 μg ml^{-1} bacterial LPS. The *P. aeruginosa* LPS at 1 μg ml^{-1} and *K. pneumoniae* LPS at 10 and 1 μg ml^{-1} caused significant enhancement of mean XTT readings ($P < 0.05$). *significant differences between mean XTT values of test and control biofilms of *C. albicans*.

Matching proteins identified in the NCBI database are shown in Table 3. Spot 2011 (A) represents a septicolysin-like protein from *C. albicans* SC5314 that is similar to *Clostridium septicum* septicolysin (Fig. 6, Table 3). A hypothetical protein CaO19.9877 from *C. albicans* SC5314 that is similar to the histidine

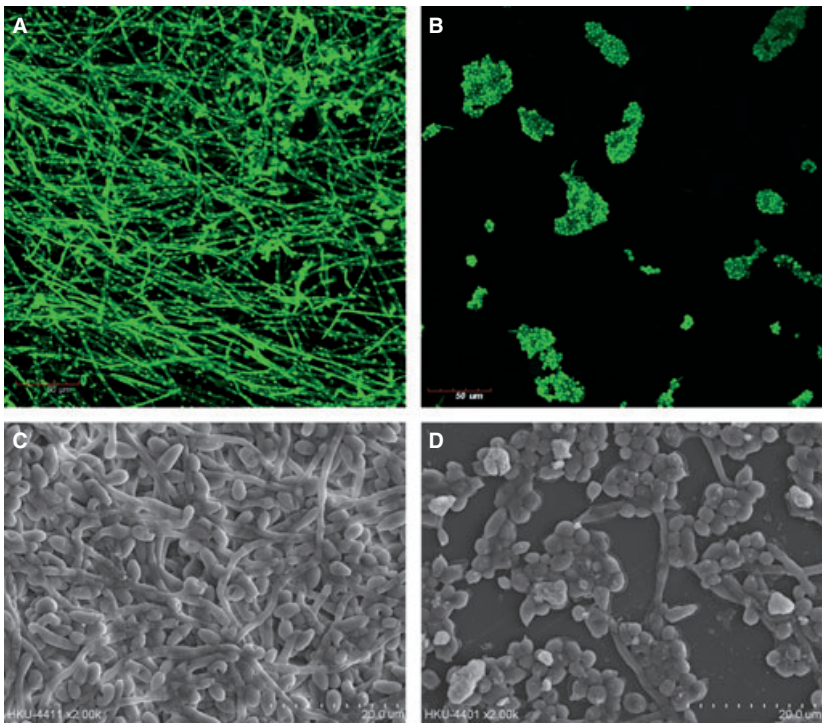

Figure 3 Ultrastructural views of *Candida albicans* SC 5314 48-h biofilms. Confocal laser scanning microscopy images of *C. albicans* biofilms (×40) (stained with Live/Dead BacLight Bacterial Viability kit; Invitrogen, Eugene, USA). Live cells are stained in green and dead cells are in red. (A) Control and (B) treated with *Pseudomonas aeruginosa* lipopolysaccharide (LPS; 100 μg ml^{-1}). Note that the LPS-treated biofilms were scantly and exclusively comprised of patchy growths of blastospores compared with well-developed control biofilm with prominent hyphae formation. (C, D) Scanning electron microscopy images of *C. albicans* biofilms (×2000) (C) Control (D) Treated with *P. aeruginosa* LPS (100 μg ml^{-1}). Note that *C. albicans* biofilm controls demonstrated densely colonized, highly organized structure with long and intermingled hyphae whereas LPS-treated biofilms showed a scanty biofilm with higher proportion of blastospores. Hyphae were fewer in number and short in the length.

triad superfamily of nucleotide-binding proteins was isolated at spot 7007 (B) (Fig. 6, Table 3). Spot 6009 (C) indicates another hypothetical protein CaO19.10301 from *C. albicans* SC5314 that is an ATP synthase d subunit involved in energy generation (Fig. 6, Table 3). We noted that spots 7004 and 7016 (D and E) represent a single protein (flavodoxin fragment of a potential reductase from *C. albicans* SC5314) that is similar to 1,4-benzoquinone reductase/flavodoxin from other fungal species (Fig. 6, Table 3). Spot 6410 (G) consisted of two proteins, namely hypothetical protein CaO19.4716 from *C. albicans* SC5314 that is similar to *Saccharomyces cerevisiae* NADP-specific glutamate dehydrogenase *GDH1*, as well as hypothetical protein CaO19.11135 of *C. albicans* SC5314, which is similar to *Saccharomyces cerevisiae* 3-phosphoglycerate kinase (*PGK1)* (Fig. 6, Table 3). Finally, spot 8411 (G) was identified as mitochondrial serine hydroxymethyltransferase (*SHMT*) from *C. albicans* (Fig. 6, Table 3).

Protein synthesis of LPS-treated *C. albicans* is affected at both the transcriptional and translational levels

The genes encoding septicolysin-like protein, and hypothetical proteins CaO19.10301, CaO19.4716 and CaO19.9877 demonstrated upregulated relative expression compared with controls by approximately 2-fold, 5-fold, 28-fold and 1.3-fold (Table 4). In contrast, genes encoding hypothetical protein CaO19.11135 and mitochondrial *SHMT* showed relative downregulation compared with control *C. albicans* biofilms by 0.2-fold and 0.7-fold, respectively (Table 4).

DISCUSSION

Previous studies have shown that biofilm-related *Pseudomonas* super-infection or co-infection can alter the pathophysiology of candidal infections leading to

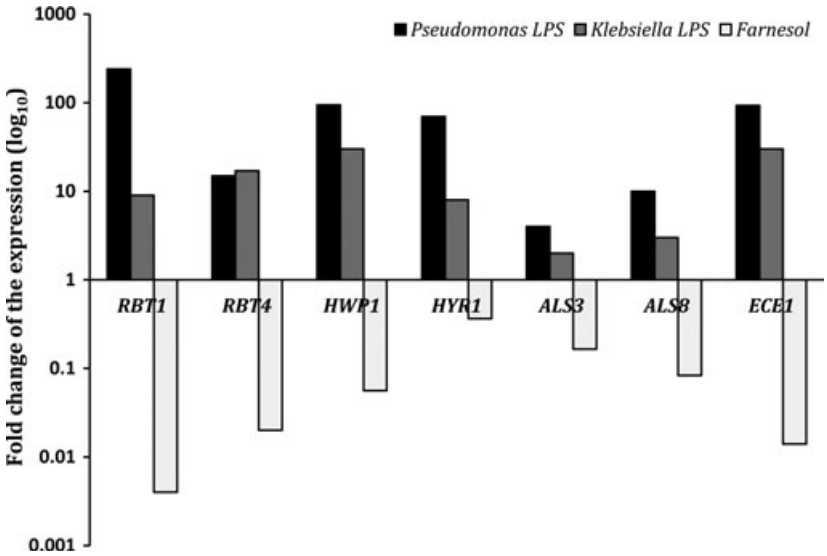

Figure 4 The mean relative expression (±SD) of *EFG1* of *Candida albicans* SC5314. The fold changes of the relative expression (±SD) of *C. albicans* SC5314 hypha-specific genes (HSGs) compared with their controls. Biofilms were treated with *Pseudomonas* and *Klebsiella* lipopolysaccharide (LPS; 100 μg ml^{-1}), and farnesol (50 μM); All HSGs showed significant upregulations in their expression when treated with LPS ($P < 0.05$). $P < 0.05$ was considered statistically significant.

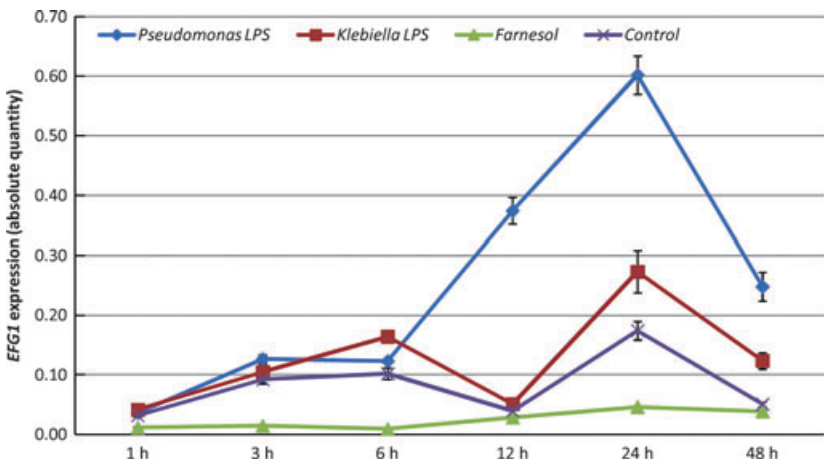

Figure 5 The mean relative expression (±SD) of *EFG1* of *Candida albicans* SC5314. The mean relative expression (±SD) of *EFG1* of *C. albicans* SC5314 in response to *Pseudomonas* and *Klebsiella* lipopolysaccharide (LPS; 100 μg ml^{-1}), farnesol (50 μM) and control at six given time points; $P < 0.05$ considered statistically significant.

serious infections such as pneumonia, and device-associated pathologies (Senpuku *et al.*, 2003; Pierce, 2005; Peres-Bota *et al.*, 2004; Bandara *et al.*, 2010b). Furthermore, our own studies indicate that various bacterial LPS have a direct modulatory effect on candidal biofilm development (Henry-Stanley *et al.*, 2003; Bandara *et al.*, 2010a). Therefore, the current study was designed to assess quantitative and qualitative effects of *P. aeruginosa* LPS on *C. albicans* biofilm development, hypha-specific gene expression, and candidal proteome.

Using the XTT reduction assay, the quantitative effects of *P. aeruginosa* and *K. pneumoniae* LPS on biofilm development of three different strains of *C. albicans* were investigated. Both types of LPS demonstrated significant stimulation of *C. albicans* biofilm development at lower concentrations of LPS (10 μg ml^{-1} and 1 μg ml^{-1}). Interestingly, at higher

Table 2 Cyclic AMP concentrations of *Candida albicans* SC5314 biofilms

	cAMP concentration (pmol ml^{-1})			
Time	*Pseudomonas* LPS	*Klebsiella* LPS	Farnesol	Control
12 h	32.9	33.4	32.9	39.2
24 h	37.6	35.9	34.4	33.9
48 h	32.9	35.9	32.4	46.1

Cyclic AMP concentrations of *C. albicans* SC5314 biofilms in response to *Pseudomonas* and *Klebsiella* lipopolysaccharide (LPS; 100 μg ml^{-1}), farnesol (50 μM) and control at 12 h, 24 h and 48 h; none of the findings were significant. $P < 0.05$ was considered statistically significant.

concentrations (100 μg ml^{-1}), the aforementioned LPS significantly suppressed biofilm formation in both the *C. albicans* clinical strain and SC5314 strain. Given that the LPS effect is concentration and strain dependent, current results further confirm the concept that bacterial LPS have a direct modulatory effect on *C. albicans* biofilm development (Bandara *et al.*, 2009, 2010a). In addition, the phenotypic variations of the bacterial LPS may affect its species-specific response (Morrison & Leive, 1975; Morrison *et al.*, 1976, 1987; Vukajlovich & Morrison, 1985; Luchi & Morrison, 2000). In contrast, Palma *et al.* (1992) noted that LPS from *E. coli*, *S. marcescens* and *Salmonella typhimurium* had no direct effect on *Candida* planktonic cultures.

The ultrastructural views of both LPS-treated and control *C. albicans* biofilms further confirm the XTT reduction assay data. In general, *C. albicans* biofilms treated with bacterial LPS were scanty and exclusively comprised blastospores whereas *C. albicans* control biofilms were relatively well defined, and confluent with a profusion of hyphae. Similar observations have been made in biofilms of various *Candida* species treated with a number of other bacterial LPS (Bandara *et al.*, 2010a).

Tandem mass spectrometric data indicated that *P. aeruginosa* LPS-treated *C. albicans* biofilm cells were deficient in several proteins that play roles in energy synthesis and metabolism, cell division and virulence. Among these proteins, we noted an interesting correlation between *PGK1* and our results (the possible associations of the other proteins with the results presented here are described in detail in the Supporting information). Recent studies have revealed that biofilm development is intrinsically associated with a hypoxic environment within it (Xu *et al.*, 1998; An & Parsek, 2007; Rossignol *et al.*, 2009). Under such oxygen stress, *Candida* biofilms appear to upregulate glycolytic pathway genes and increase glycolytic flux to compensate for the defect in energy synthesis via the respiratory chain (Garcia-Sanchez *et al.*, 2004; Murillo *et al.*, 2005; Yeater *et al.*, 2007; Askew *et al.*, 2009; Ernst & Tielker, 2009; Rossignol *et al.*, 2009). Bonhomme *et al.* (2011) noted that glycolytic genes are capable of controlling the expression of hypha-specific genes (Fig. S1). Our results further indicate that *PGK1,* which catalyses a crucial step in the glycolytic pathway in production of ATP

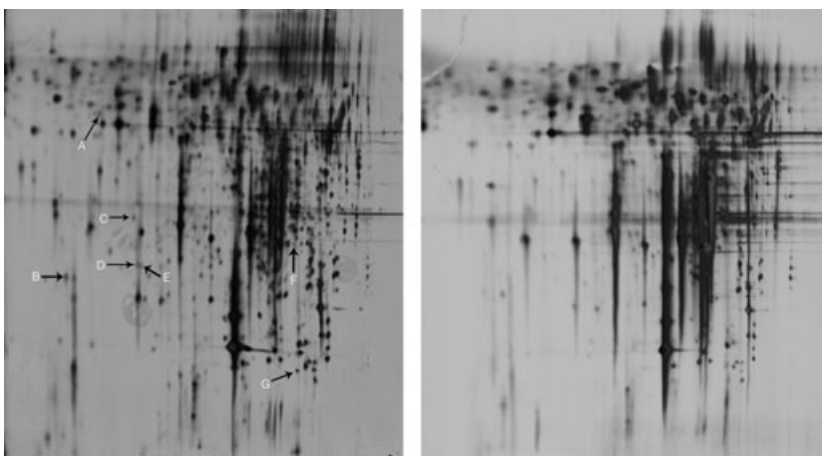

Figure 6 Two-dimensional gel electrophoresis image of *Candida albicans* SC5314 biofilms for 18 h. Left panel: Control; Right panel: Test. Protein spots that were absent in *Pseudomonas* lipopolysaccharide (LPS; 100 μg ml^{-1}) treated sample. (A) Spot No.2011, (B) Spot No.7007, (C) Spot No.6009, (D) Spot No. 7004, (E) Spot No.7016, (F) Spot No.6410 and (G) Spot No.8411.

Table 3 Mass spectrometric data

Spot no.	Protein	Matched identity	Gene	Nominal mass (M_r)	pI value	Matched peptides	Sequence coverage (%)	*P* value
2011	Septicolysin-like protein [*Candida albicans* SC5314]	gi|68485961	CaO19.8335	19007	5.31	49	29	<0.05
6009	Hypothetical protein CaO19.10301 [*Candida albicans* SC5314]	gi|68488805	*ATP7*	19364	6.19	48	28	<0.05
6410	Hypothetical protein CaO19.4716 [*Candida albicans* SC5314]	gi|68491798	*GDH1*	49888	5.73	150	32	<0.05
	Hypothetical protein CaO19.11135 [*Candida albicans* SC5314]	gi|68489602	*PGK1*	45266	6.07	89	21	<0.05
7004	Potential reductase, flavodoxin fragment [*Candida albicans* SC5314]	gi|68482400	CaO19.2241	14380	8.71	9	7	>0.05
7007	Hypothetical protein CaO19.9877 [*Candida albicans* SC5314]	gi|68484224	*HNT1*	17089	6.35	53	34	<0.05
7016	Potential reductase, flavodoxin fragment [*Candida albicans* SC5314]	gi|68482400	CaO19.2241	14380	8.71	9	7	>0.05
8411	Serine hydroxymethyltransferase, mitochondrial OS = *Candida albicans* GN = SHM1 PE = 3 SV = 1	GLYM_CANAL	*SHM1*	54588	9.00	107	21	<0.05

Proteins that were not expressed in *Pseudomonas aeruginosa* lipopolysaccharide-treated *Candida albicans* SC5314 biofilms compared with its control.

Table 4 Real-time polymerase chain reaction data

Spot no.	Protein		C_T Mean	Men normalized quantity	Mean normalized SD	Relative quantity	Relative quantity min	Relative quantity max
2011	Septicolysin-like protein	Control	23.39	1.26	1.03	1.00	0.94	1.06
	[*Candida albicans* SC5314]	Test	23.18	2.71	1.06	2.15	1.87	2.46
6009	Hypothetical protein CaO19.10301	Control	22.92	1.03	1.03	1.00	0.93	1.07
	[*Candida albicans* SC5314]	Test	21.52	5.10	1.02	4.93	4.64	5.25
6410	Hypothetical protein CaO19.4716	Control	21.84	1.04	1.05	1.00	0.89	1.13
	[*Candida albicans* SC5314]	Test	17.49	29.51	1.03	28.19	26.00	30.56
	Hypothetical protein CaO19.11135	Control	15.80	1.11	1.05	1.00	0.88	1.13
	[*Candida albicans* SC5314]	Test	18.89	0.25	1.03	0.23	0.21	0.22
7007	Hypothetical protein CaO19.9877	Control	21.17	0.97	1.03	1.00	0.94	1.06
	[*Candida albicans* SC5314]	Test	21.66	1.30	1.16	1.34	0.94	1.91
8411	Serine hydroxymethyltransferase,	Control	23.05	1.06	1.05	1.00	0.89	1.12
	mitochondrial OS = *Candida*	Test	24.47	0.75	1.14	0.71	0.51	0.97
	albicans GN = SHM1 PE = 3 SV = 1							

The relative expression of the genes that encoded proteins unexpressed in *Pseudomonas aeruginosa* lipopolysaccharide-treated *Candida albicans* SC5314 biofilms compared with its control.

(Mazzoni *et al.*, 2009) (the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate), is totally suppressed by *P. aeruginosa* LPS, likely resulting in defective glycolysis in *C. albicans* cells (Fig. S1). As a consequence, it appears that there is a significant upregulation of all the HSGs (Fig. S1). Real-time PCR data further suggested that the suppression of *PGK1* occurs at the gene level as *PGK1* expression was significantly downregulated when treated with LPS.

Interestingly, in hypoxic conditions, *EFG1* suppresses filamentation (Doedt *et al*., 2004) and stimulates glycolytic genes to facilitate adaptation to hypoxia (Setiadi *et al*., 2006) (Fig. S1). Therefore, yeast to hypha transition and the expression of HSGs in *C. albicans* under hypoxia are *EFG1* independent (Brown *et al*., 1999; Sonneborn *et al*., 1999; Setiadi *et al*., 2006; Ernst & Tielker, 2009) (Fig. S1). In this study, we observed that *EFG1* was significantly upregulated throughout the course of *C. albicans* biofilm formation. On the other hand, *P. aeruginosa* LPS had no significant effect on cAMP levels in *C. albicans* cells, further validating our hypothesis that the cAMP/PKA signaling pathway is not involved in the regulation of HSGs. As yeast to hypha transition under a hypoxic environment is *EFG1* independent, it is likely that the primary role of *EFG1* in *C. albicans* biofilms treated with *P. aeruginosa* LPS must be to act as a hypoxic response regulator, and upregulate the glycolytic pathway.

According to real-time PCR data, the relative expression of the genes encoding the aforementioned proteins confirmed that both gene and protein expression of *PGK1* and its protein and *SHMT* and its protein were well correlated. In contrast, the rest of the genes demonstrated converse patterns of expression from their proteins (NADP-dependent glutamate dehydrogenase 1, septicolysin-like protein, mitochondrial ATPase d subunit and Hnt1). Although it is too early too put forward a clear-cut explanation for such differences, it is tempting to suggest that *Pseudomonas* LPS may affect post-transcriptional/translational modifications of these genes or may structurally or functionally alter the final protein product.

In summary, the current study implies that LPS has significant and direct modulatory effects on *C. albicans* biofilm development. Under the hypoxic condition of the biofilm environment, *Pseudomonas* LPS suppresses *C. albicans* glycolysis. Low levels of cellular ATP stimulate hyperfilamentation and HSGs. The hypoxic environment elevates *EFG1* transcription, which stimulates glycolysis and suppresses HSGs. The oxygen stress causes *EFG1*-independent pathways to stimulate HSG expression. The final outcome of HSG expression and filamentation would be downstream signaling of the *EFG1*-dependent and -independent pathways. Further molecular work on biofilm hypoxia and mutant phenotypes is necessary to evaluate the response of *C. albicans* to *P. aeruginosa* LPS and to explain the molecular mechanisms underlying the species-specific response of *C. albicans* to LPS during biofilm formation, and to evaluate the role of LPS as a putative antifungal agent and biomimetic molecule.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Mr. Simon Lee for technical help and Dr. O.L.T Lam for editorial assistance. This study was supported by grant CERG HKU 7624/06M from The University of Hong Kong.

REFERENCES

An, D. and Parsek, M.R. (2007) The promise and peril of transcriptional profiling in biofilm communities. *Curr Opin Microbiol* **10**: 292–296.

Askew, C., Sellam, A., Epp, E. *et al.* (2009) Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*. *PLoS Pathog* **5**: e1000612.

Aurell, C.A. and Wistrom, A.O. (1998) Critical aggregation concentrations of gram-negative bacterial lipopolysaccharides (LPS). *Biochem Biophys Res Commun* **253**: 119–123.

Azoulay, E., Timsit, J.F., Tafflet, M. *et al.* (2006) *Candida* colonization of the respiratory tract and subsequent *Pseudomonas* ventilator-associated pneumonia. *Chest* **129**: 110–117.

Bamford, C.V., d'Mello, A., Nobbs, A.H., Dutton, L.C., Vickerman, M.M. and Jenkinson, H.F. (2009) *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. *Infect Immun* **77**: 3696–3704.

Bandara, H.M., Yau, J.Y., Watt, R.M., Jin, L.J. and Samaranayake, L.P. (2009) *Escherichia coli* and its lipopolysaccharide modulate *in vitro Candida* biofilm formation. *J Med Microbiol* **58**: 1623–1631.

Bandara, H.M., Lam, O.L., Watt, R.M., Jin, L.J. and Samaranayake, L.P. (2010a) Bacterial lipopolysaccharides variably modulate *in vitro* biofilm formation of *Candida* species. *J Med Microbiol* **59**: 1225–1234.

Bandara, H.M., Yau, J.Y., Watt, R.M., Jin, L.J. and Samaranayake, L.P. (2010b) *Pseudomonas aeruginosa* inhibits *in-vitro Candida* biofilm development. *BMC Microbiol* **10**: 125.

Bonhomme, J., Chauvel, M., Goyard, S., Roux, P., Rossignol, T. and d'Enfert, C. (2011) Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm

formation by *Candida albicans*. *Mol Microbiol* **80**: 995–1013.

Brown, D.H. Jr, Giusani, A.D., Chen, X. and Kumamoto, C.A. (1999) Filamentous growth of *Candida albicans* in response to physical environmental cues and its egulation by the unique *CZF1* gene. *Mol Microbiol* **34**: 651–662.

De Castro, C., Parrilli, M., Holst, O. and Molinaro, A. (2010) Microbe-associated molecular patterns in innate immunity: extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. *Methods Enzymol* **480**: 89–115.

Doedt, T., Krishnamurthy, S., Bockmuhl, D.P. *et al.* (2004) APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. *Mol Biol Cell* **15**: 3167–3180.

Douglas, L.J. (2003) *Candida* biofilms and their role in infection. *Trends Microbiol* **11**: 30–36.

Ernst, J.F. and Tielker, D. (2009) Responses to hypoxia in fungal pathogens. *Cell Microbiol* **11**: 183–190.

Federle, M.J. and Bassler, B.L. (2003) Interspecies communication in bacteria. *J Clin Invest* **112**: 1291–1299.

Garcia-Sanchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J.M. and d'Enfert, C. (2004) *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* **3**: 536–545.

Henry-Stanley, M.J., Hess, D.J., Erickson, E.A., Garni, R. M. and Wells, C.L. (2003) Effect of lipopolysaccharide on virulence of intestinal *Candida albicans*. *J Surg Res* **113**: 42–49.

Hogan, D.A., Vik, A. and Kolter, R. (2004) A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol* **54**: 1212–1223.

Hogenauer, C., Hammer, H.F., Krejs, G.J. and Reisinger, E.C. (1998) Mechanisms and management of antibiotic-associated diarrhea. *Clin Infect Dis* **27**: 702–710.

Jin, Y., Samaranayake, L.P., Samaranayake, Y. and Yip, H.K. (2004) Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol* **49**: 789–798.

Jin, Y., Zhang, T., Samaranayake, Y.H., Fang, H.H., Yip, H.K. and Samaranayake, L.P. (2005) The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms. *Mycopathologia* **159**: 353–360.

Klotz, S.A., Gaur, N.K., De Armond, R. *et al.* (2007) *Candida albicans* Als proteins mediate aggregation with bacteria and yeasts. *Med Mycol* **45**: 363–370.

Leroy, O., Gangneux, J.P., Montravers, P. *et al.* (2009) Epidemiology, management, and risk factors for death of invasive *Candida* infections in critical care: a multicenter, prospective, observational study in France (2005–2006). *Crit Care Med* **37**: 1612–1618.

Levison, M.E. and Pitsakis, P.G. (1987) Susceptibility to experimental *Candida albicans* urinary tract infection in the rat. *J Infect Dis* **155**: 841–846.

Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**: 999–1007.

Luchi, M. and Morrison, D.C. (2000) Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of gram-negative bacteria. *Infect Immun* **68**: 1899–1904.

MacFarlane, T.W. (1990) Ecology and epidemiology of *Candida*. In: Samaranayake, L.P., MacFarlane, T.W. eds. *Oral Candidosis*. London: Wright, pp. 21–46.

Mazzoni, C., Torella, M., Petrera, A., Palermo, V. and Falcone, C. (2009) *PGK1*, the gene encoding the glycolitic enzyme phosphoglycerate kinase, acts as a multicopy suppressor of apoptotic phenotypes in *S. cerevisiae*. *Yeast* **26**: 31–37.

Miceli, M.H., Diaz, J.A. and Lee, S.A. (2011) Emerging opportunistic yeast infections. *Lancet Infect Dis* **11**: 142–151.

Morales, D.K. and Hogan, D.A. (2010) *Candida albicans* interactions with bacteria in the context of human health and disease. *PLoS Pathog* **6**: e1000886.

Morrison, D.C. and Leive, L. (1975) Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. *J Biol Chem* **250**: 2911–2919.

Morrison, D.C. and Ryan, J.L. (1987) Endotoxins and disease mechanisms. *Annu Rev Med* **38**: 417–432.

Morrison, D.C., Betz, S.J. and Jacobs, D.M. (1976) Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. *J Exp Med* **144**: 840–846.

Morrison, D.C., Vukajlovich, S.W., Ryan, J.L. and Levin, J. (1987) Structural requirements for gelation of the *Limulus* amebocyte lysate by endotoxin. *Prog Clin Biol Res* **231**: 55–73.

Murillo, L.A., Newport, G., Lan, C.Y., Habelitz, S., Dungan, J. and Agabian, N.M. (2005) Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* **4**: 1562–1573.

Palma, C., Cassone, A., Serbousek, D., Pearson, C.A. and Djeu, J.Y. (1992) Lactoferrin release and interleukin-1, interleukin-6, and tumor necrosis factor production by human polymorphonuclear cells stimulated by various lipopolysaccharides: relationship to growth inhibition of *Candida albicans*. *Infect Immun* **60**: 4604–4611.

Peres-Bota, D., Rodriguez-Villalobos, H., Dimopoulos, G., Melot, C. and Vincent, J.L. (2004) Potential risk factors for infection with *Candida spp.* in critically ill patients. *Clin Microbiol Infect* **10**: 550–555.

Pfaller, M.A., Diekema, D.J., Gibbs, D.L. *et al.* (2007) Results from the ARTEMIS DISK Global Antifungal Surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J Clin Microbiol* **45**: 1735–1745.

Pierce, G.E. (2005) *Pseudomonas aeruginosa, Candida albicans*, and device-related nosocomial infections: implications, trends, and potential approaches for control. *J Ind Microbiol Biotechnol* **32**: 309–318.

Ramage, G., Vandewalle, K., Wickes, B.L. and Lopez-Ribot, J.L. (2001) Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* **18**: 163–170.

Ramage, G., Saville, S.P., Thomas, D.P. and Lopez-Ribot, J.L. (2005) *Candida* biofilms: an update. *Eukaryot Cell* **4**: 633–638.

Rietschel, E.T., Schade, U., Jensen, M., Wollenweber, H. W., Luderitz, O. and Greisman, S.G. (1982) Bacterial endotoxins: chemical structure, biological activity and role in septicaemia. *Scand J Infect Dis Suppl* **31**: 8–21.

Rossignol, T., Ding, C., Guida, A., d'Enfert, C., Higgins, D. G. and Butler, G. (2009) Correlation between biofilm formation and the hypoxic response in *Candida parapsilosis*. *Eukaryot Cell* **8**: 550–559.

Samaranayake, L.P., (2002) Fungi of relevence to dentistry. In: Samaranayake L. P. ed. *Essential Microbiology for Dentistry*. London: Churchill livingstone, pp. 142–147.

Schaller, M., Schafer, W., Korting, H.C. and Hube, B. (1998) Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microbiol* **29**: 605–615.

Senpuku, H., Sogame, A., Inoshita, E., Tsuha, Y., Miyazaki, H. and Hanada, N. (2003) Systemic diseases in association with microbial species in oral biofilm from elderly requiring care. *Gerontology* **49**: 301–309.

Setiadi, E.R., Doedt, T., Cottier, F., Noffz, C. and Ernst, J. F. (2006) Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. *J Mol Biol* **361**: 399–411.

Shirtliff, M.E., Krom, B.P., Meijering, R.A. *et al.* (2009a) Farnesol-induced apoptosis in *Candida albicans*. *Antimicrob Agents Chemother* **53**: 2392–2401.

Shirtliff, M.E., Peters, B.M. and Jabra-Rizk, M.A. (2009b) Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* **299**: 1–8.

Sonneborn, A., Tebarth, B. and Ernst, J.F. (1999) Control of white-opaque phenotypic switching in *Candida albicans* by the Efg1p morphogenetic regulator. *Infect Immun* **67**: 4655–4660.

Vukajlovich, S.W. and Morrison, D.C. (1985) Activation of murine spleen cells by lipid A: negative modulation of lipid A mitogenic activity by O-antigen polysaccharide. *J Immunol* **135**: 2546–2550.

Wang, J., Ohshima, T., Yasunari, U. *et al.* (2006) The carriage of *Candida* species on the dorsal surface of the tongue: the correlation with the dental, periodontal and prosthetic status in elderly subjects. *Gerodontology* **23**: 157–163.

Weber, K., Schulz, B. and Ruhnke, M. (2010) The quorum-sensing molecule *E,E*-farnesol – its variable secretion and its impact on the growth and metabolism of *Candida* species. *Yeast* **27**: 727–739.

Xu, K.D., Stewart, P.S., Xia, F., Huang, C.T. and McFeters, G.A. (1998) Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* **64**: 4035–4039.

Yeater, K.M., Chandra, J., Cheng, G. *et al.* (2007) Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* **153**: 2373–2385.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Tables S1 and S2. The Mean XTT values (±SD) of *Candida albicans* control and test biofilms treated with *Pseudomonas aeruginosa* (S1) and *Klebsiella pneumoniae* (S2) lipopolysaccharide in a concentration gradient for 48 h.

Table S3. The quantification of hypha-specific gene expression in *Candida albicans* SC5314.

Table S4. The quantification of *EFG1* expression in *Candida albicans* SC5314.

Figure S1. Possible relationships of the effect of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) on *Candida albicans* biofilms and protein expression.

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